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THE ROLE OF PALATINE TONSILS IN THE DEVELOPMENT OF STREPTOCOCCUS SUIS INFECTION IN PIGS

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Role of the palatine tonsils in the development of *Streptococcus suis* infection in pigs.

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Introduction

Streptococcus suis (*S.suis*) infection remains a significant cause of disease and economic loss within the pig industry worldwide, through deaths, lameness and cost of antibiotics used in control. This industry is undergoing major expansion in Saskatchewan at the present time and given the current inadequate understanding of the pathogenesis of this disease and inability to erradicate the carrier state, *S.suis* infection will continue to be an important disease. Intensive research has been directed at elucidating its virulence factors and their mode of action, and no single factor seems to hold all the clues. A recent review highlights the lack of progress despite major efforts in different laboratories both in N. America and Europe (Gottschalk & Segura, 2000) .

Project:

This project was designed with the objective of answering some of the unresolved questions associated with this disease, with particular focus on the role of the palatine tonsils in the pathogenesis of S.suis infection. The tonsils are surface (mucosa)-associated lymphoid organs (MALT) which take up pathogenic organisms and other material from the oral cavity and serve both as a portal of entry of infection, and also as a carrier site for organisms without causing disease. Being lymphoid organs and part of the immune system, they participate in defence and form the first major barrier of the upper alimentary tract. *S.suis* has been shown to gain entry through the tonsils but studies focusing on the interaction between the host animal and the organism are few. The crypt epithelium is extensively infiltrated by leucocytes, mainly lymphocytes, and also by varying numbers of neutrophils, and is closely applied to the underlying lymphoid tissue which, apart from lacking afferent lymphatics i.e. lymph vessels draining into the tissue, has many features in common with lymph nodes. Bacteria and viruses, both virulent and avirulent, can reach this lymphoid tissue from the oral cavity only by first crossing the lympho-epithelial barrier of the crypts.

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Objective1:
To examine the relationship between the epithelium and the leucocytes of the lymphoid tissue, particularly those invading the epithelium in an *in vitro* study system.

Methods to be used were dependent on successful establishment of tonsillar coculture systems and organ culture of tonsillar tissue. As explained in an earlier report, this work had to be set aside as the lymphocytes consistently died very early into the culture period, and despite seeking expert advice from Dr S.Federoff, Professor Emeritus of Anatomy, College of Medicine, U of S, we were unable to overcome this problem. Viability of lymphocytes which is dependent on many co-stimulatory molecules may be difficult to achieve in a tissue/explant culture system even in the short term of a few days. A second complicating factor was that in order to eliminate the normal commensal flora found in the tonsil, heavy use of antibiotic was required, in essence rendering the system unsuitable for bacterial studies. Thirdly the tissue structure was found to be too severely comprised to yield useful results from electron-microscopic studies.

It was therefore decided to concentrate on *in vivo* experiments, using pigs and *S.suis*.

Objective 2:
To examine the relationship between the epithelium and the cells of the lymphoid tissue, particularly those invading the epithelium, using *Streptococcus suis* in pigs following experimental infection *in vivo*.

The methods chosen for these studies were electron microscopy (EM), confocal microscopy (CM), immuno-histochemistry (IHC) and reverse transcriptase-polymerase chain reaction (RT-PCR).

Ultimately, only the IHC and EM studies were fruitful.

RESULTS:

Confocal microscopy:
CM was dependent on achieving expression of the jellyfish green fluorescent protein (GFP) gene in a genetically modified strain of *S.suis* type 2 being developed at VIDO in Dr.P.Willson's laboratory. Gene expression had been successful in *E.coli*, a Gram-negative organism; generally, however, Gram-positive bacteria, which include *S.suis*, are less easily manipulated, perhaps due to the capsular properties in these organisms, and only partial insertion was occurred, and expression of GFP and associated fluorescence was not achieved. The organism was therefore unsuitable for CM studies which were dependent on the expression of fluorescence. The gene for resistance to the antibiotic Kanamycin was however successfully inserted and this property was useful in the recovery of the experimental strain from infected animals.

Immuno-histochemistry:

Identification of immune cell populations in tonsillar crypt epithelium.
In the literature on tonsils of pigs, the cells which perform immune functions i.e. the white blood cell (leukocyte) populations have been characterised quantitatively only and no data exists regarding distribution of these cells *in situ*. Knowing the distribution is important, since as indicated above, there are distinct functional areas with the tonsils, namely 1) the crypt epithelium which is the zone of first exposure and defence and is extensively infiltrated by cells which participate in defence and immune activities , and 2) the lymphoid tissue which is more analogous to a lymph node. In the intestine it is known that intraepithelial immune cells belong to specific subpopulations not typically found in other lymphoid tissue.

We characterised the immune cells in the crypt epithelium immunohistochemically using a panel of specific monoclonal antibodies. The presence of specific subsets of T cells, B cells, macrophages and neutrophils was identified and then analysed quantitatively, using a combination of human (for delineation of fields of crypt epithelium to be captured) and digital analysis of the various populations within the captured images of tonsillar epithelium.

1. Our initial study characterised the leukocytes in crypt epithelium of the tonsils of normal healthy market weight pigs. We investigated specific lymphocyte subpopulations in the tonsillar crypt epithelium of ten adult healthy pigs, using monoclonal antibodies against T lymphocyte markers CD3, CD4, CD8, gd T cell receptor, and a B cell marker (immunoglobulin light-chain) using IHC (avidin-biotin immunoperoxidase technique). The crypt epithelium was usually extensively infiltrated by different T cell populations and by B cells. Each subset was variable among animals and within individual animals. CD4 T cells and the gamma-delta TCR cells predominated over CD8 Tcells.

   These results were reported by Dr Monica Salles, Ph.D. student working on this project, in an oral presentation at the 10th Interrnational Congress of Mucosal Immunology in June 1999 in Amsterdam in the Netherlands and have also been published (Salles & Middleton, 2000). This represents the first documentation of these cell populations correlated with specific distribution in the tonsillar crypt epithelium of pigs and it permits preliminary basic conclusions to be drawn regarding their function in the epithelium. That both T cells and B cells are present in the crypt epithelium indicates that this epithelium is capable of participating in both cellular (T cells) and humoral (B cells) immune responses. The subset of CD4 T cells predominates over the CD8 T cell subset. Gamma-delta T cells represent a large proportion of the T cells, suggesting that in pigs,, these cells have an important role in mucosal defence and immunity.

   A significant finding emerging from these studies is that there is considerable variation within individual pigs in the different cell populations and also within

the crypt epithelium from one crypt unit to another within the same pig, suggesting that each unit within the tonsils responds independently to its own local environment and bacterial flora. The palatine tonsils of the pig have ~180 crypts.

2. The second study involved a short term experimental infection with *S. suis,* carried out in conjunction with Dr P. Willson at VIDO. Here we investigated the early changes, from 18h to 72h, that occur in leucocyte subpopulations of the crypt epithelium of the palatine tonsils of 3 week-old pigs in response to *S. suis* type 2 infection. Monoclonal antibodies against leucocyte markers CD3, CD4, CD8, gamma-delta T cell receptor, lamda-immunoglobulin light-chain, myeloid cells, and major histocompatibility complex class II molecule (MHC-II) were used for IHC (avidin-biotin immunoperoxidase technique). For this work pigs were euthanised at 18, 24, 48 and 72 hours following intra-oral infection of the tonsils with *S. suis.* Tonsillar samples from infected and non-infected control animals were examined culturally and also by IHC for *S. suis.*, and by IHC for leucocyte populations.

a) Correlating with the last point in (1) above, in pigs infected orally with *S. suis*, the bacteria were found to be randomly distributed amongst the different crypt units in the tonsils of individual pigs. This corresponded to observations in previous studies done by the principal investigator. The tonsils of pigs or any other species are not sterile under normal conditions and are infected with a wide range of different bacteria and viruses, both pathogenic (i.e. potentially disease-causing) and non-pathogenic. It is therefore likely that these also have a random distribution throughout the tonsils, influencing the defence and immune functions in each individual crypt unit.

b) Digital analysis was carried out similarly on these to determine if infection with *S. suis* was reflected in changes in these immune cell populations on the front line of defence within the crypt epithelium. For each cell type the sections analysed were stained in series with immediately adjacent sections stained for *S.suis* so that for each cell type there was a direct correlation with the *S.suis* infected portions of crypts.

An increase in the number of lamda-immunoglobulin light-chain positive cells (B cell subset) occurred in crypt epithelium of *S. suis* infected animals from 18h after infection onwards. This was significant at 18h and 48h after infection when compared to uninfected control animals. CD4 and CD8 Tcells increased from 18h onwards, with a significant increase at 24h and 72h post-infection. No significant difference cells between infected and control animals, however, was detected for CD3 T cells, cells with the gamma-delta T cell receptor, or for MHC-II cells.

Macrophages, neutrophils and crypt epithelial cells stained positively with the myeloid marker. Positive MHC-II staining in the crypt epithelium was

increased in infected animals, with a significant difference detected at 24h and 72h after infection.

These studies revealed that statistically significant quantitative changes primarily involving cells of the innate immune system i.e. neutrophils, and macrophages, and in specific T cell subsets, and the changes varied with the time post-infection. Taken together, these results suggest that in the early phase of *S. suis* infection there is participation of the innate immunity, represented by neutrophils, macrophages and likely epithelial cells, and that there is a potential for the initiation of both humoral and cellular responses against *S. suis* within the crypt epithelium of the palatine tonsil.While not all populations showed statistically significant numerical differences, qualitative changes such as changes in cytokine expression remain possible and could lead to significant alterations in the immune response, and may explain the lack of change in the T cell subset with the gamma-delta receptor. An increase in this subset had been anticipated as it is thought to be important in young pigs prior to the full maturation of other subsets.

The amount of *S.suis* material increased in the crypt epithelium over the time frame of the experimental infection but no corresponding major increase was seen in the lymphoid tissue below. This suggests that mechanisms exist in the epithelial barrier to limit the amount of *S.suis*, either as whole bacteria or fragments, that reaches the lymphoid tissue. It may be that disease results when these mechanisms are disabled by some means e.g. by the effects of stress on the animal (this remains a somewhat ill-defined though real factor), or perhaps by concurrent infection such as a viral infection, or disturbances in the commensal flora of the tonsils.

These studies were accepted for presentation in the poster session at the 6th International Veterinary Immunology Symposium, 2001, in Sweden, and have been accepted for publication (Salles, Perez-Casal, Willson & Middleton, *2002*) . They constitute the first documentation of the cellular responses *in situ* to *S.suis* infection in pigs and studies extending over a longer time period will be required to evaluate fully the nature of the immune response evoked by *S.suis* infection.

Electron-microscopy:

Methods were successfully developed and applied to detect *S. suis* immunohistochemically in tissue processed for electron-microscopy (EM). Given the random and unpredictable distribution of the bacteria and the requirement for very small pieces of tissues in EM work, this technique permitted screening of tissue blocks and selection of positive material for examination by EM. Bacteria consistent with *S. suis* were identified within three different cells types - epithelial cells, neutrophils & mononuclear phagocytes. This correlated well with the results of IHC, confirming that these cells have an important role in the early stage of infection in ingesting the organism and perhaps in presenting it and its antigens to the lymphoid tissue below. Further, an immunogold technique was successfully adapted to identify specifically *S.suis* Type 2 (the organism used to infect the pigs) by EM. This work formed the basis of a poster presentation at the annual Canadian Society of Microbiology meeting to be held in Winnipeg in June, 2000 (Salles, Willson & Middleton) and a paper for publication is presently under revision.

EM studies demonstrated cells with morphology suggestive of dendritic cells (long processes which extended between crypt epithelial cells) which are a specific category antigen presenting cells. The identity and function of these cells requires further study.

Cytokine analysis:

Cytokines are the messenger molecules which enable cells of the immune system to "talk" and give instructions to each other, and depending on specific cell function, their production may be either up- or down-regulated in infectious disease, inflammatory and immune reactions. *S.suis* has been shown to stimulate production of cytokines by murine macrophages (Segura *et al.*1999) and it is to be expected that a similar response occurs in the pig which is the natural host.

Initially it was proposed to use RT-PCR for this analysis but as *in situ* hybridisation (ISH), a technique had been used successfully in Dr. John Gordon's laboratory for cytokine detection (nterleukin-8 in cattle) it was considered more likely to be successful. Both techniques are very challenging technically. Unfortunately at the present time few antibodies to the important porcine cytokines have become available for veterinary research as these could have been tried in IHC, a technique which works well for us.

A revised proposal was submitted and accepted for this portion of the work involving studies on cytokines.

First it was necessary to produce the primers required for identification of key sequences within the messenger RNA molecule involved in the expression of each of the cytokine genes. Considerable effort went into this with much welcome assistance from Dr. Greg Appleyard of the PCR laboratory in the Departments of

Veterinary Microbiology and Pathology. Using specific sequences, fresh pig DNA and PCR, we attempted numerous times to obtain the required sequences for the primers for a variety of cytokines. Except for tumour necrosis factor-alpha (TNF-α), each time that the apparently correct and pure product was obtained, sequencing (carried out at the NRC Plant Biotechnology lab. on campus) proved inconsistent with published sequences for these molecules and were unsuitable to carry forward to the next stage. Approximately 800 PCR reactions were carried out in the process of trying to achieve successful results, along with very many variations in the thermocycler program and concentration of salt solutions, in order to try to optimise the conditions. Purification using plasmids was also attempted but to no avail.

A DNA probe was successfully developed for the kanamycin gene in S.suis and while this proved positive in the test development stage, when tried in ISH expression was not detected in sections of *S.suis* infected tonsils, and similarly no expression of TNF-α using ISH could be obtained. Since positive controls do not exist for this sytem, it is not possible to know if the method failed to work, if the method was insufficiently sensitive to detect expression, or if there was no expression there to detect. Multiple attempts were made to demonstrate *S.suis* and TNF-α using the ISH technique. Material requested from Dr.B.Wilke's lab. in Guelph did not survive the shipment process and was not re-shipped, despite further request.

Summary and Conclusions:

This project investigated the interaction of a natural pathogen of pigs, *Streptococcus suis* Type 2, with the cells in the crypt epithelium of the palatine tonsils of pigs. The tonsils form the first line of defence by the immune system against organisms gaining entry to the body through the mouth. The extensive open meshwork of the crypt epithelium, a "lining" which is infiltrated by various leucocyte populations and subsets, is where the disease-causing organisms first encounter the cells of the immune system.

Leucocyte subsets were defined qualitatively and quantitatively by immunohistochemistry in the crypt epithelium of market weight pigs, and in weaned piglets experimentally infected with S.suis. In both groups of pigs, considerable variations in the various subset populations occurred among pigs and also interestingly among crypts within the same pig, suggesting that each crypt is a distinct unit capable of responding somewhat independently to its own local environment and infectious agents. *S.suis* was seen to have a random and unpredictable distribution among the crypt units within any one infected pig and such variation in distribution of pathogens may explain the differences seenn cell complement within the epithelium among different crypts. Statistically significant differences occurred in specific T cell subsets. In response to *S.suis* there was an increase in the number of neutrophils and macrophages in the epithelium and of CD4 and CD8 T cells.

The intraepithelial presence of T cells and B cells indicates that the tonsillar crypt epithelium is capable of participating in both cellular and humoral immune responses.

Although the number of animals in this study was small, important observations were made in relation to the *S. suis* infection at the level of the crypt epithelium of the palatine tonsil. Cells of the innate immune system, i.e. neutrophils, macrophages, and likely epithelial cells, participate in the initial phase of *S. suis* infection. An increase in specific lymphocyte subsets within the first 24h post-infection suggest a potential for the initiation of humoral and cellular responses in the crypt epithelium. For other populations present in the epithelium, participation in the early detection of *S. suis* by secretion of cytokines that recruit other cells cannot be ruled out. Also, in this epithelium, gamma-delta T cells are not the dominant T cell subset, unlike in the peripheral blood of 4 week-old pigs.

In vitro studies on tonsils as tissue and organ cultures proved impractical for a number of reasons. Technical approaches to demonstrating expression of cytokines (the messenger molecules of cell cross-talk and instruction) in response to S.suis infection were extremely challenging and at the present time have not been successful.

Electron microscopy was used to determine which cells in the crypt interact with *S.suis* in the early period of an infection. Neutrophils and macrophages were seen to engulf organisms, as did epithelial cells. This correlated well with the increases seen in these two leucocyte populations associated with experimental infection.

Future directions:
To obtain full understanding of the role of the tonsils in infectious disease, future studies on cytokine production in response to infection and linked to identification of the specific cells responsible for producing the cytokines are likely to be helpful. In particular results of such studies may aid in clarifying why some animals may become carriers without developing disease and why others do not, and why a carrier may convert into a clinical case under certain conditions such as stress. Novel avenues to prophylaxis may be uncovered.

Development of antibodies to a wide range of porcine cytokines will greatly enhance progress in this area. At the International Veterinary Immunology meeting in Sweden, it was clear that veterinary researchers are presently hampered by the lack of available antibodies to porcine cytokines and it is hoped that collaborative efforts among different laboratories will see production and availability increase in the next few years. Such progress will greatly enhance the capability for studying cytokines in tissues and for determining their role in the pathogenesis of infectious diseases of pigs.

Future studies on tonsillar function in the pathogeneis of disease could be done using a Gram negative pathogen of pigs such as *Actinobacillus pleuropneumoniae* inserted with GFP gene, as a model of bacterial infection of the tonsil, since this bacterium is frequently harboured in the tonsils. The potential major advantage of using bacteria with the GFP gene is that tracking the bacteria in successive generations over time becomes possible.

References:
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Segura,M. Stankova,J. & Gottschalk, M. 1999. Heat-killed *Streptococcus suis* capsular type 2 strains stimulate tumour necrosis factor alpha and interleukin-6 in murine macrophages. Infect.Immun. 67: 4646-4654.

Publications arising from this work:

Salles, M.W.S. & Middleton,D.M. 2000. Lymphocyte subsets in porcine tonsillar epithelium. Vet.Immunol.Immunopathol. 77:133 -134.

Salles,M.W.S., Perez-Casal,J., Willson,P.& Middleton, D.M. Vet. Immunol. Immunopathol. (Accepted 2002)

Other avenues of dissemination of results:

6th International Veterinary Immunology Symposium, 2001
Canadian Society of Microbiologists,2000
10th International Conference of Mucosal Immunology, The Netherlands, 1999
Saskatchewan Pork Industry Symposium, Saskatoon. 1999
Life Sciences Poster Day (Salles) Univ. of Saskatchewan, 2000
WCVM Graduate Student Poster Day (Salles), 2001, 2002